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Triplex-induced Recombination in Human Cell-free Extracts

DEPENDENCE ON XPA AND HsRad51*

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Triple helix-forming oligonucleotides (TFOs) can bind to polypurine/polypyrimidine regions in DNA in a sequence-specific manner. Triple helix formation has been shown to stimulate recombination in mammalian cells in both episomal and chromosomal targets containing direct repeat sequences. Bifunctional oligonucleotides consisting of a recombination donor domain tethered to a TFO domain were found to mediate site-specific recombination in an intracellular SV40 vector target. To elucidate the mechanism of triplex-induced recombination, we have examined the ability of intermolecular triplexes to provoke recombination within plasmid substrates in human cell-free extracts. An assay for reversion of a point mutation in the *supFG1* gene in the plasmid pSupFG1/G144C was established in which recombination in the extracts was detected upon transformation into indicator bacteria. A bifunctional oligonucleotide containing a 30-nucleotide TFO domain linked to a 40-nucleotide donor domain was found to mediate gene correction *in vitro* at a frequency of 46×10^{-5}, at least 20-fold above background and over 4-fold greater than the donor segment alone. Physical linkage of the TFO to the donor was unnecessary, as co-mixture of separate TFO and donor segments also yielded elevated gene correction frequencies. When the recombination and repair proteins HsRad51 and XPA were depleted from the extracts using specific antibodies, the triplex-induced recombination was diminished, but was either partially or completely restored upon supplementation with the purified HsRad51 or XPA proteins, respectively. These results establish that triplex-induced, intermolecular recombination between plasmid targets and short fragments of homologous DNA can be detected in human cell extracts and that this process is dependent on both XPA and HsRad51.

Targeted modification of the genome by gene replacement is of value as a research tool and has potential application to gene therapy. However, although facile methods exist to introduce new genes into mammalian cells, the frequency of homologous integration is limited (1), and isolation of cells with site-specific gene insertion typically requires a selection procedure (2). Site-specific DNA damage in the form of double-strand breaks produced by rare cutting endonucleases can promote homologous recombination at chromosomal loci in several cell systems (3–7), but this approach requires the prior insertion of the recognition sequence into the locus. Because intermolecular triple helices can provoke DNA repair (8), oligonucleotide-mediated triple helix formation has been proposed as a potentially more general approach to sensitizing a target site to homologous recombination (9–12).

TFOs[1] can bind in the major groove of DNA to polypurine/polypyrimidine sequences, forming specific Hoogsteen or reverse-Hoogsteen hydrogen bonds with the purine strand of the duplex (13, 14). Triplex formation has been shown to inhibit transcription in mammalian cells (15) and can be used to deliver a DNA-reactive conjugate to a specific target site both in complex DNA mixtures *in vitro* (16, 17) and within mammalian cells in culture (18–22), in some cases leading to site-directed mutations (19, 20). Triplex formation, by itself, can be mutagenic, and evidence suggests that the nucleotide excision repair (NER) and transcription-coupled repair pathways may play a role in the triplex-induced mutagenesis (8).

In previous work, we found that triple helix-directed psoralen cross-links could stimulate recombination in a plasmid substrate containing two tandem copies of the *supFG1* reporter gene (9). Subsequent work established that triplex formation, even in the absence of covalent DNA damage, could stimulate recombination between repeated sequences, an effect that was absent in cells deficient in the NER factor, XPA (10). Recent work has extended these findings to the demonstration of TFO-induced recombination at a chromosomal locus containing two tandem copies of the herpes simplex virus thymidine kinase gene, following direct intranuclear microinjection of the oligonucleotides (11).

Based on the ability of TFOs to mediate specific molecular recognition of a DNA target site within a cellular genome and on the observation that triplex formation can stimulate recombination, we also tested a series of bifunctional oligonucleotides consisting of a TFO designed to bind to bp 167–196 of the *supFG1* reporter gene coupled to a short (40 nt) segment of DNA homologous to bp 121–160 of the gene. Such a hybrid molecule, designated a tethered donor-TFO (TD-TFO), was found to mediate recombination with the *supFG1* gene present in an SV40-based episomal vector in monkey cells at frequencies in the range of 0.1–1% (Ref. 23 and data not shown), demonstrating that TFOs can promote *inter*molecular as well as *intra*molecular recombination in mammalian cells. This result is consistent with studies demonstrating that bifunctional

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[1] The abbreviations used are: TFO, triple helix-forming oligonucleotide; NER, nucleotide excision repair; nt, nucleotide(s); bp, base pair(s); DTT, dithiothreitol; TD, tethered donor; HPLC, high pressure liquid chromatography.

oligonucleotides can mediate both triplex formation and strand invasion on plasmid substrates *in vitro* (24, 25).

In the present study, we have used a plasmid-based assay to investigate triple helix-induced recombination in human cell-free extracts. We find that triple helix formation can stimulate recombination between a plasmid and short homologous fragments *in vitro*. Stimulation was observed whether or not the donor fragment was directly linked to the TFO. Recombination was reduced in the absence of the TFO as well as when the TFO was substituted with a non-triplex-forming, scrambled sequence oligonucleotide. To probe the mechanism of the induced recombination, the roles of the NER damage recognition factor, XPA (26), and the human recombinase, HsRad51 (27), were directly tested by experimental manipulation of the respective protein levels in the extracts, either via immunodepletion with specific antibodies or supplementation with purified proteins. We report here that both XPA and HsRad51 are required for triple helix-induced recombination, and that increased HsRad51 levels can boost the efficiency of the reaction.

EXPERIMENTAL PROCEDURES

Plasmid Vector—The shuttle vector plasmid pSupFG1/G144C, containing a *supFG1* gene with an inactivating G:C to C:G point mutation at position 144, was described previously (23).

Oligonucleotides—Oligonucleotides were synthesized by the Midland Certified Reagent Co. (Midland, TX) and purified by either gel electrophoresis or high pressure liquid chromatography (HPLC), followed by Centricon-3 filtration in distilled water (Amicon, Beverly, MA). The oligonucleotides consisted primarily of phosphodiester linkages but were modified at the 3′ end to resist exonuclease activity by the inclusion of phosphorothioate linkages at the terminal three residues. In the TD-TFO molecule (designated A–AG30), the linker segment between the donor fragment and the TFO domain consisted of the sequence 9TT9TT9, in which 9 indicates a 9-atom polyethylene glycol linker (Spacer 9, Glen Research, Sterling, VA). The specific TFO, designated AG30, has the sequence 5′-AGGAAGGGGGGGTGGTGGGGGAGGGGGAG-3′ and is designed to bind as a third strand to bp 167–176 of the *supFG1* gene. The donor domain (A) consists of a 40-nt synthetic single-stranded DNA fragment homologous to positions 121–160 of the *supFG1* gene (5′-AGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGG-3′). The scrambled sequence oligonucleotide, SCR30, has the same base composition as AG30 but differs at 12 positions: 5′-GGAGGAGTGGAGGGGAGTGAGGGGGGGGG-3′.

Cells—Construction of *E. coli* SY302 *lacZ125*(Am) *recA56 hsdR2*::Tn*10* trp-49 has been described previously (28). HeLa cells were maintained and grown by the National Cell Culture Center (Minneapolis, MN) and were obtained as cell pellets for extract preparation.

Proteins and Antibodies—HsRad51 protein was purified from *E. coli* DH10B (Life Technologies, Inc.) carrying plasmid pEG932. Purification consisted of chromatography through Q Sepharose, Bio-Gel-http, Mono-Q, and native DNA-cellulose. Other purification details have been reported previously (29). Purification was documented by SDS-polyacrylamide gel electrophoresis analysis, yielding a single visualized band of 37 kDa following the DNA-cellulose purification step. Purified HsRad51 protein was injected into rabbits to produce high affinity polyclonal antibodies specific to the HsRad51 protein (29).

XPA protein was produced using an *Escherichia coli* expression vector (obtained from R. Wood) containing the human XPA cDNA sequence, along with an N-terminal 6-histidine tag, in the pET-15b plasmid (Novagen, Madison, WI). The protein was expressed in an *E. coli* expression strain, BL21(DE3) pLysS, as described by Jones and Wood (30). Following expression, XPA was purified using immobilized metal-affinity chromatography (Talon resin; CLONTECH, Palo Alto, CA) under native conditions. Fractions were eluted with a buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, and 100 mM imidazole. Western analysis was used to determine specificity using antibodies to the 6-His tag. Coomassie staining was used to determine purity. To further confirm that the expressed and purified protein was the correct species and present as soluble monomers in solution, XPA protein was subjected to both mass spectrometry and HPLC size exclusion chromatography/laser light scattering analysis. The results revealed a monodispersed peak at a molecular mass of 36.8 kDa, indicating a monomer of the correct size. Rabbits were immunized with the purified XPA protein (100 μg/injection) to produce high affinity antibodies specific to XPA.

Preparation of Cell-free Extract—HeLa whole cell extract was prepared as described previously (28). Briefly, HeLa cells were washed with phosphate-buffered saline and resuspended in 0.01 M Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM DTT, followed by lysis using a Dounce homogenizer. The lysate was diluted in four packed cell volumes of 0.05 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 2 mM DTT, 25% sucrose, 50% glycerol, and a protease inhibitor mixture (Sigma catalog no. P8340). One packed cell volume of saturated $(NH_4)_2SO_4$ (0.33 g/ml of solution) was added and then neutralized by 1 N NaOH, followed by centrifugation at 15,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 0.025 M HEPES, pH 7.9, 0.1 M KCl, 0.012 M $MgCl_2$, 0.05 mM EDTA, 2 mM DTT, 17% glycerol and was dialyzed in the same buffer for 8–12 h. The sample was quick frozen in liquid N_2 and stored at −80 °C. The preparation typically contained 15–20 mg of protein/ml.

In Vitro Assay for Recombination—Reactions consisted of 3 μg of pSupFG1/G144C plasmid DNA, 3 μg each of selected oligonucleotides (TFO, donor fragment, or both), 60 mM NaCl, 2 mM β-mercaptoethanol, 3 mM KCl, 12 mM Tris-HCl, pH 7.4, 2 mM ATP, 0.1 mM each dNTPs, 2.5 mM creatine phosphate, 1 μg of creatine phosphokinase, 12 mM $MgCl_2$, 0.1 mM spermidine, 2% glycerol, 0.2 mM DTT, and 15–20 μl of cell-free extract in a 50-μl total reaction volume. After incubating 2 h at 30 °C, the reactions were terminated by the addition of 25 μM EDTA, 0.5% SDS, and 20 μg of proteinase K. After incubation at 37 °C for 1 h, the plasmid DNA was isolated by phenol extraction and ethanol precipitation and dissolved in 10 μl of H_2O. 1 μl of the resulting sample was used to transform *E. coli* SY302 by electroporation, as described (28), followed by growth of the cells on indicator plates for genetic analysis of *supFG1* gene function as described previously (19).

Depletion of HsRad51 and XPA from Cell Extracts—Anti-HsRad51 or anti-XPA sera were adjusted to 1× Tris-buffered saline (10 mM Tris-HCl, pH-7.5, 100 mM NaCl) and then incubated with pre-swollen Protein A-Sepharose beads for 1 h at 4 °C. The beads were washed three times with Tris-buffered saline buffer and incubated with 50 μl of HeLa cell extract for 2 h on ice with gentle rotation. The supernatant (HsRad51- or XPA-depleted extract) was recovered by centrifugation and subsequently examined by Western blot and used in the *in vitro* recombination assay.

Solubilization of RAD51 Immunoprecipitates—After incubating the cell extract with HsRad51 antibody-Protein A-Sepharose beads, the beads were centrifuged and washed twice with 10 mM phosphate buffer, pH 7.2. Then one bead volume of 100 mM glycine, pH 2.5, and 3.5 M $MgCl_2$ was added and kept at 4 °C. After 1 h, the sample was centrifuged and the supernatant was immediately dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.4, 0.5 mM DTT, 0.2 mM EDTA, 10% glycerol, and 100 mM KCl. The supernatant was directly used to supplement the depleted extracts for the recombination assay without freezing.

RESULTS

Experimental Design—The substrate for triplex-targeted recombination was the plasmid vector pSupFG1/G144C, containing a mutated version of the *supFG1* amber suppressor tRNA gene, *supFG1*-144, which has an inactivating G:C to C:G mutation at bp 144. The function of this gene can be readily assayed in indicator bacteria carrying an amber stop codon in the *lacZ* gene, and so *supFG1*-144 is a useful reporter of recombination events that revert the gene to the functional sequence. The *supFG1*-144 gene also contains a 30-bp, G-rich site at the 3′ end of the gene to which the G-rich 30-mer TFO (AG30) can bind to form a triple helix in the anti-parallel motif (Fig. 1).

In a strategy to promote targeted recombination, we designed a TD-TFO molecule (A–AG30) in which the AG30 TFO is tethered to a donor DNA fragment homologous to a region of the *supFG1*-144 target gene via a mixed sequence linker (Fig. 1) (23). This arrangement facilitates target site recognition via triple helix formation while at the same time positioning the donor fragment for possible recombination and information transfer. This strategy also is intended to exploit the ability of a triple helix, itself, to provoke DNA repair, potentially increasing the probability of recombination with the homologous donor DNA. In the bifunctional A–AG30 molecule, the donor fragment, A, consists of a single-strand of length 40 synthesized to be homologous to positions 121–160 of the *supFG1*-144 gene

except at position 144, where the sequence matches that of the functional *supFG1* gene.

Triplex-induced Recombination in HeLa Cell-free Extracts—In previous work, we demonstrated the occurrence of triplex-induced recombination upon transfection of A–AG30 into monkey COS cells already carrying the pSupFG1/G144C vector as an episomal, SV40-replicon-based target (23). To investigate the mechanism of triplex-induced recombination, in the present work we have tested the ability of triplex formation to promote recombination within human cell-free extracts.

Selected oligonucleotides were incubated with the target pSupFG1/G144C vector in HeLa whole cell extracts supplemented with nucleotides and ATP. Following a 2-h incubation, the plasmid vector DNA was isolated and used to transform *recA*, *lacZ*(Amber) indicator *E. coli* to score for *supFG1* gene function (Fig. 2). The results show that the bifunctional oligonucleotide, A–AG30, was active in the extracts and produced *supFG1*-144 gene reversion at a frequency of 45×10^{-5}. Note that this effect occurred in the extract and was not mediated by recombination in the indicator bacteria because, without incubation in the extract, no recombinant products were observed upon transformation of the A–AG30 sample into bacteria. The A donor fragment was also somewhat active, as co-mixture of A plus the pSupFG1/G144C plasmid led to a low level of *supFG1* reversion, consistent with the ability of short fragments of DNA to mediate recombination and marker rescue (31–33). However, the effect of A–AG30 was 4-fold higher than that of A alone, demonstrating the influence of the TFO domain and providing direct evidence for triplex-induced recombination *in vitro*. By itself, however, the TFO domain produced minimal reversion over background, indicating the need for the sequence information provided by the A donor fragment.

Interestingly, the sample in which AG30 and the A donor oligonucleotide were not linked but were simply co-mixed as separate molecules together with the plasmid substrate also produced an increased level of recombination, at a frequency of 40×10^{-5}, almost as high as that produced by the linked A–AG30. This result provides further evidence that a TFO can stimulate recombination between a donor fragment and a target locus. In addition, because the donor fragment in this case is separate from the TFO, the result specifically demonstrates a role for the TFO in stimulating recombination that is distinct from its ability to deliver a tethered donor fragment to the target site.

In another sample tested, the A donor was linked to an oligonucleotide segment designated SCR30, consisting of the same base composition as AG30 but a scrambled sequence creating 12 mismatches. SCR30 does not bind to the *supFG1* gene and so does not form a triplex. It also has no homology to the target gene. Linkage of SCR30 to the donor fragment was found to actually inhibit recombination relative to the donor fragment alone.

Role of HsRad51 in Triplex-induced Recombination—The results above establish that triplex-induced recombination can be reconstituted in HeLa cell extracts *in vitro*. By using this *in vitro* system, we sought to determine the role(s) of selected recombination and repair proteins in the pathway of triplex-induced recombination. HsRAD51 is a human recA homolog that functions in homologous recombination and has been shown to mediate DNA pairing and strand exchange reactions (34). To test the role of HsRAD51 protein in this homologous gene conversion, we used a polyclonal rabbit anti-HsRad51 antibody to deplete HsRad51 protein from the cell extract. Successful depletion of HsRad51 from the extract was confirmed by Western blot (Fig. 3). The depleted extract was tested for the ability to support triplex-induced recombination (Table I), both in the case of the linked A–AG30 bifunctional molecule and in the case of the co-mixed separate A and AG30 sample. Immunodepletion of HsRad51 was found to substantially reduce the frequency of recombinants in both cases. Control samples demonstrated that Protein A-Sepharose, in the absence of the HsRad51 polyclonal antibody, had no effect.

Limited Complementation by Addition of Purified HsRad51 to the HsRad51-depleted Cell Extract—Next, we tested the extent to which the triplex-induced recombination in the HsRad51-depleted extracts could be restored by the addition of

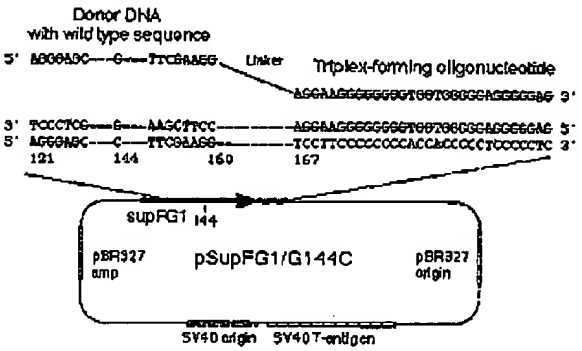
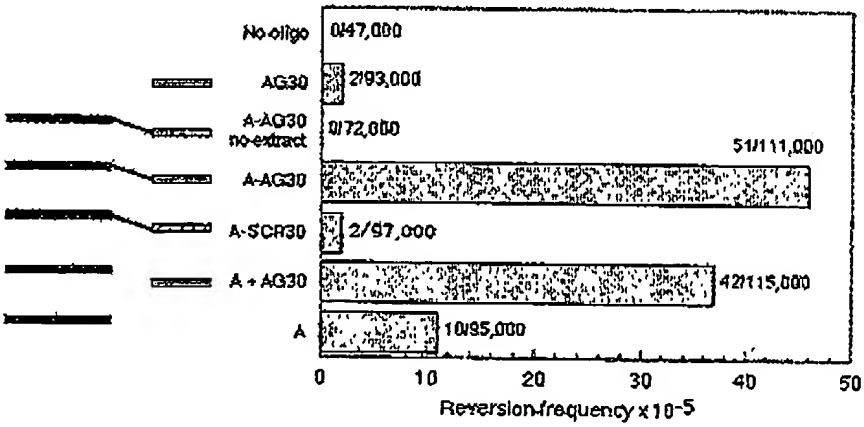


FIG. 1. **Schematic diagram depicting the binding of the AG30 TFO to the *supFG1*-144 gene in the vector pSupFG1/G144C.** AG30 in this example is linked to a 40-nt donor DNA fragment homologous to bp 121–160 of the *supFG1*-144 gene. The G at position 144 of the donor fragment is intended to correct the inactivating G:C to C:G mutation at bp 144 in the target gene. The linker segment consists of the sequence, 9TT9TT9, where "9" indicates a 9-atom polyethylene glycol linker. In some experiments, the donor fragment and the TFO were used separately or were co-mixed but not linked.

FIG. 2. **Triplex-induced recombination in human cell-free extracts.** The pSupFG1/G144C plasmid DNA was incubated *in vitro* with the indicated oligonucleotides in the presence or absence of HeLa whole cell extracts. After 2 h, the plasmid DNA was isolated and used to transform indicator bacteria for genetic analysis of the *supFG1* gene. A schematic dingram of each oligonucleotide or oligonucleotide combination is presented to the *left*. *Plus* sign (+) indicates that the different oligonucleotides are mixed together but unlinked. *Minus sign* (–) indicates that the various oligonucleotides are connected. The *bars* indicate the frequency of blue colonies (representing recombinants) out of the total colonies, with the actual count given to the *right* of each *bar*. The results are cumulative data from three experiments.



purified, recombinant HsRad51 protein. Increasing amounts of HsRad51 protein were added to the depleted extracts, and the recombination assay was carried out (Table II). Even after the addition of a large amount of HsRAD51 (up to 5 μg), only a portion of the triplex-induced recombination activity was recovered. We hypothesized that the lack of complementation by purified HsRad51 might reflect the removal from the immunodepleted extracts of other factors physically associated with HsRad51. To test this, we supplemented the immunodepleted extracts with re-solubilized HsRad51 immunoprecipitate (Table II). Addition of the solubilized immunoprecipitate to the depleted extracts was found to almost completely restore the recombination activity, indicating that HsRad51 immunodepletion removes more than HsRad51 alone and that HsRad51 supplementation, by itself, cannot compensate for the loss of the other factors. This result is not surprising in light of emerging evidence that the recombination complex in human cells consists of multiple factors, including Rad52, Rad54, XRCC2, and XRCC3, as well as members of the RAD51 family, including Rad51B/Rad51L1, Rad51C/Rad51L2, and Rad51D/Rad51L3 (35, 36).

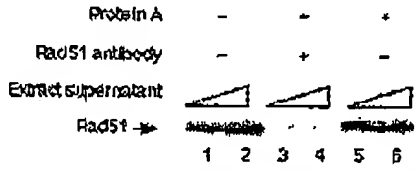


FIG. 3. **Immunodepletion of HsRad51 from the HeLa cell extracts.** Extract samples were immunodepleted using a polyclonal HsRad51 antibody pre-mixed with Protein A-Sepharose beads. The immunoprecipitate was removed by centrifugation, and the remaining supernatant was examined by Western blot analysis. Samples were treated as indicated.

Addition of HsRad51 to the Complete Extract Boosts Activity—Increasing amounts of HsRAD51 (from 250 ng to 2 μg) were added to non-depleted whole cell extracts, and triplex-induced recombination was measured (Table III). Both in the case of the linked donor fragment and TFO (A−AG30) and the unlinked donor plus TFO (A+AG30), additional HsRad51 was found to increase the frequency of the triplex-induced recombinants. In the samples supplemented with amounts of HsRad51 in the lower range, there was a minimal effect. However, at higher levels of supplementation, increased yields of recombinants

TABLE I

Effect of HsRad51 depletion on triplex-induced recombination in cell-free extracts

The results represent the combined data from three independent experiments.

Oligonucleotide	Extract treatment	Blue colonies/total	Reversion frequency
			× 10^{-6}
None	Standard	0/110,000	0
A−AG30	No extract	0/95,000	0
A−AG30	Standard	53/99,000	54
A−AG30	Protein A-Sepharose	45/93,000	48
A−AG30	HsRad51 antibody	9/110,000	8
A+AG30	Standard	42/105,000	40
A+AG30	Protein A-Sepharose	36/96,000	38
A+AG30	HsRad51 antibody	9/99,000	9

TABLE II

Effect of HsRad51 supplementation on triplex-induced recombination in HsRad51-depleted cell-free extracts

The results represent the combined data from three independent experiments.

Oligonucleotide	Extract treatment	Blue colonies/total	Reversion frequency
			× 10^{-6}
None	Standard	0/111,000	0
A−AG30	No extract	0/99,000	0
A−AG30	Standard	51/112,000	46
A−AG30	HsRad51 antibody	12/112,000	11
A−AG30	HsRad51 antibody + 500 ng of HsRad51	12/117,000	10
A−AG30	HsRad51 antibody + 1 μg of HsRad51	15/105,000	14
A−AG30	HsRad51 antibody + 2 μg of HsRad51	18/112,000	16
A−AG30	HsRad51 antibody + 5 μg of HsRad51	21/114,000	18
A−AG30	HsRad51 antibody + 10 μl of solubilized HsRad51 immunoprecipitate	40/108,000	37
A−AG30	HsRad51 antibody + 20 μl of solubilized HsRad51 immunoprecipitate	46/109,000	42

TABLE III

Effect of HsRad51 supplementation on triplex-induced recombination in cell-free extracts

The results represent the combined data from three independent experiments.

Oligonucleotide	Extract treatment	Blue colonies/total	Reversion frequency
			× 10^{-6}
None	Standard	0/93,000	0
A−AG30	No extract	0/87,000	0
A−AG30	Standard	54/110,000	49
A−AG30	Plus 250 ng of HsRad51	43/95,000	45
A−AG30	Plus 500 ng of HsRad51	55/98,000	56
A−AG30	Plus 1 μg of HsRad51	79/105,000	75
A−AG30	Plus 2 μg of HsRad51	103/97,000	106
A+AG30	No extract	0/93,000	0
A+AG30	Standard	39/105,000	37
A+AG30	Plus 500 ng of HsRad51	37/110,000	34
A+AG30	Plus 1 μg of HsRad51	54/114,000	47
A+AG30	Plus 2 μg of HsRad51	76/102,000	75

were seen. Hence, even though HsRad51, by itself, cannot fully complement the activity of the immunodepleted extracts, it can provide increased activity to otherwise complete whole cell extracts.

The Role of the Nucleotide Excision Repair Factor, XPA—In previous work studying TFO-induced mutagenesis and recombination within SV40 vectors in human cells, we had obtained genetic evidence that the ability of triplex formation to stimulate DNA metabolism is dependent on the activity of the NER pathway (8, 10). To obtain direct biochemical evidence in support of this proposed mechanism, we tested the requirement for the NER damage recognition factor, XPA (26), in the triplex-induced recombination in the HeLa cell extracts.

A rabbit polyclonal antibody was raised against recombinant human XPA protein produced in *E. coli* and was found to recognize a single protein in human cells of the expected size (data not shown). Using this antibody, XPA was removed from the extracts by immunoprecipitation. Depletion of XPA was confirmed by Western blot analysis of the residual samples (Fig. 4). Depletion of XPA from the extracts was found to substantially reduce the frequency of TFO-induced recombination, whether or not the TFO was covalently linked to the donor fragment (Table IV). With both the A−AG30 and the A+AG30 samples, the depletion of XPA reduced the frequency of recombinants to that mediated by the donor fragment alone (Table I). Hence, the ability of a triple helix to stimulate recombination depends on the XPA protein. This result supports the hypothesis that the NER pathway can recognize a triple helix as a "lesion," thereby provoking DNA metabolism that can lead to recombination or mutation.

Following XPA immunodepletion, we tested the ability of XPA to restore the triplex-induced recombination activity (Table IV). The results show that increasing amounts of XPA protein provide functional complementation in the depleted extracts. These results establish a direct role for XPA in mediating the ability of a triple helix to stimulate recombination.

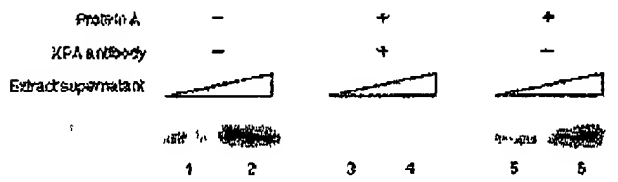


FIG. 4. **Immunodepletion of XPA protein from the HeLa cell extracts.** Extract samples were immunodepleted using a polyclonal XPA antibody pre-mixed with Protein A-Sepharose beads. The immunoprecipitate was removed by centrifugation, and the remaining supernatant was examined by Western blot analysis. Samples were treated as indicated.

DISCUSSION

The work reported here establishes that triplex-induced recombination can be detected in human cell-free extracts. A 30-mer TFO that binds with high affinity to a portion of the *supFG1* reporter gene within the pSupFG1/G144C vector was found to stimulate recombination between the vector and a 40-nt donor fragment. Recombination was induced both when the donor fragment was linked to the TFO and when it was present as a separate, unlinked molecule. The stimulation was determined to occur in the extracts and not in the indicator bacteria because no recombinants were observed unless the samples were incubated in the extracts. The donor fragment, by itself, was able to participate in recombination with the plasmid in the extracts, consistent with previous studies that have detected intermolecular recombination in similar mammalian cell extracts (37). However, the present work establishes that such intermolecular recombination can be stimulated by third strand binding to one of the molecules.

Establishment of TFO-induced recombination allowed testing of the role of selected factors in the process. Immunodepletion of HsRad51, a human recombinase homologous to recA (34), from the extract reduced the yield of induced recombinants, but purified HsRad51 did not fully compensate for the immunodepletion. When the immunoprecipitate was re-solubilized and used to supplement the depleted extracts, the induced recombination activity was restored. These results suggest that the immunoprecipitate contains factors in addition to HsRad51 that are essential for the reaction. Such factors could include HsRad51-associated proteins that are proposed to play a role in homologous recombination, such as HsRad51a, HsRad51b, HsRad52, HsRad54, XRCC2, and XRCC3 (35, 36). On the other hand, the addition of extra HsRad51 to the non-depleted extracts produced an increased frequency of recombinants, suggesting that HsRad51, itself, plays a critical role in the process. This result is consistent with the observation that overexpressed HsRad51 can provide a modest increase in the frequency of recombination in reporter gene substrates in mammalian cells (38).

The NER damage recognition factor, XPA, was also found to play an essential role in the triplex-induced recombination, as no induced recombinants were seen in the extracts after XPA immunodepletion. Supplementation of the induced extracts with recombinant XPA protein restored the induced recombination activity. This result not only is consistent with previous work showing that triplex-induced mutagenesis and recombination are substantially reduced in human mutant cell lines deficient in XPA (8, 10), it also demonstrates directly that XPA is required for the process. Taken together, the data support a model in which the oligonucleotide-mediated triple helix is recognized by XPA, thereby initiating repair activity that can create recombinogenic intermediates. Such intermediates may

TABLE IV

Effect of XPA depletion on triplex-induced recombination in cell-free extracts

The results represent the combined data from three independent experiments.

Oligonucleotide	Extract treatment	Blue colonies/total	Reversion frequency
			$\times 10^{-6}$
None	Standard	0/209,000	0
A−AG30	No extract	0/213,000	0
A−AG30	Standard	96/219,000	44
A−AG30	Protein A-Sepharose	45/114,000	39
A−AG30	XPA antibody	18/214,000	8
A−AG30	XPA antibody + 75 ng of XPA	27/112,000	24
A−AG30	XPA antibody + 150 ng of XPA	42/114,000	37
A−AG30	XPA antibody + 300 ng of XPA	45/118,000	38
A+AG30	Standard	46/118,000	39
A+AG30	Protein A-Sepharose	39/111,000	35
A+AG30	XPA antibody	12/115,000	10

either be correctly repaired, repaired with incorporation of mutation in an error-prone manner, or, if homologous DNA is present, serve as substrates for repair by a HsRad51-dependent pathway of homologous recombination.

In the extracts, the TFO was found to sensitize the plasmid to recombination either with a linked donor fragment or with an unlinked fragment, at approximately the same frequency. This ability of the TFO to stimulate recombination between the target site and an unlinked fragment is in contrast to a previous study examining TFO-induced recombination in COS cells, in which the linked TD-TFO molecule was found to be 4-fold more active than the mixture of the unlinked molecules (23). In that study, the pSupFG1/G144C vector was pre-transfected into COS cells, and the cells were transfected the next day with the oligonucleotides. Two days later, the vector DNA was isolated for analysis in indicator bacteria. We interpret this difference between the previous cell study and the present work to suggest that, in the *in vitro* reactions, the TFO and donor fragment are present in adequate local concentrations whether or not they are linked together covalently. Hence, in the extracts, the ability of the TFO to deliver a linked donor fragment to the target site and place it in juxtaposition with the region of homology is not as important in promoting recombination as is the ability of the triplex to provoke DNA metabolism. In contrast, in the cell experiments, both properties of the TFO appear to be needed, although it remains to be determined whether the need for linkage of the donor to the TFO in cells can be overcome by increasing the efficiency of donor fragment transfection. If so, it would allow a gene correction strategy in which a TFO could be used in combination with larger donor fragments, greater than those that can be synthesized in continuity with the TFO. This would be advantageous, since previous studies examining recombination between episomal or chromosomal targets and transfected DNAs in mammalian cells have consistently shown that fragments in the range of 500 bp or larger produce higher levels of recombination than do short fragments in the size range used here (31–33, 39, 40).

Overall, the work reported here demonstrates that triplex-induced recombination can be detected in human cell free extracts, and it provides insight into the underlying mechanism by identifying critical roles for HsRad51 and XPA. The results suggest a pathway of triplex-induced recombination that depends on NER and on homologous recombinational repair of NER-generated intermediates. The ability to reconstitute TFO-induced recombination *in vitro* should serve as a basis for further elucidation of the manner in which triplex formation can provoke DNA metabolism, and may thereby guide refinements in strategies to use TFOs to promote targeted genetic changes in human cells.

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